Final Report

For the period 07/1999 to 06/2001

Richard Stohlman Award in Breast Cancer Prevention

A Postdoctoral Fellowship from the Cancer Research Foundation of America

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Project Title

"Gene Expression Profiling for High-Throughput Screening of Human Tumors"

Contents:

Attachment: reprints of published journal articles 1-7 and award publication.

A. Research Results -- development of a cDNA microarray platform for tyrosine kinase gene expression profiling in human cancers

1. Background and motivation

Tyrosine kinases (tk) belong to a family of proteins that function in signal transduction to regulate cell growth and survival. The expression of about 100 such tk genes is carefully orchestrated in normal cellular development while dis-regulation of some of these tk genes often leads to uncontrolled cell growth that eventually turns into malignant tumors. Well-known examples of this phenomenon include the erbB2 over-expression in breast cancer and the BCR/ABL chimeric protein in chronic myeloid leukemia. Previously, investigation of tk gene expression could be done only for one or two genes at a time. Recent technological advances have led to the development of a new technology - cDNA microarrays - which allows simultaneous monitoring of the expression of thousands of genes in one experiment.

The P.I.'s lab has been studying the abnormal expression of tk genes and their association with tumorgenesis and the progression of cancer. In recent years, researchers in the lab isolated more than 50 tk genes expressed in tumors of the thyroid and breast. With many tk genes isolated and cDNA microarray technology in hand, it was possible to devise a system that enabled us to monitor the expression of multiple tk genes in breast cancers.

2. A microarray system for tyrosine kinase gene expression profiling

Prior to the commencement of his research supported by the Richard Stohlman Award in Breast Cancer Prevention and provided by the Cancer Research Foundation of America, Dr. Hsieh was involved in the DNA microarray project with Dr. Weier for nearly a year. He constructed a DNA microarrayer system that can be used to deposit thousands of different DNA molecules onto glass slides pre-coated with poly-L-lysine. This coating provides a positively charged layer to tightly bind DNA to the slides.

The Weier laboratory has assembled an extensive panel of cloned tyrosine kinase genes fragments. Sizes and identities of the inserts were verified by agarose gel electrophoresis and PCR amplification with tk-specific primers. For cDNA microarray fabrication, each tk insert product is isolated after PCR amplification and concentrated by isopropanol precipitation. A fifty-percent mixture of H_2O and dimethyl sulfoxide (DMSO) or Arrayit solution (Telechem International, San Jose, CA) is used to dissolve DNA at a concentration above 125 ng/µl. The addition of DMSO/Arrayit solution produced spots that were more homogeneous and maintained consistent diameters. The concentration of DNAs used for arraying was maintained at 125ng/µl to ensure that a sufficient amount was retained after post-printing slide processing. The total number of known and novel tk clones included in the panel and arrayed on the slides is 60. A typical printing session spots 60 tk genes and controls in duplicate onto 50 slides (2 arrays per slide) in approximately 4 hours. The printed slides were subjected to post-printing processing including UV cross-linking, blocking, denaturation, and dehydration to produce arrays ready for hybridization.

Quality control of the printed arrays was performed through visual inspection and DNA staining with fluorescent dyes as well as test hybridization. Our laboratory has a Zeiss Axioplan II Mot fluorescence microscope equipped with computer-controlled X,Y,Z stages, 2 filter changers, a Hamamatsu digital camera as well as the KS300 software running on a Pentium 233 MMX computer. This microscope was mainly used for probe visualization and image acquisition for fluorescence in situ hybridization (FISH). It could be converted into an array image acquisition workstation for microarray quality control purposes. The KS 300 software provides microscope system control via an interactive menu with graphic user interface and allows user-programmable macros. With a 10X objective lens, the system is capable of acquiring images of $1mm^2$. At a 100µm spot diameter and a 200µm pitch, 16 images (4x4) cover an array of 144 spots (12x12). A macro was written by Dr. Hsieh for the KS300 software to allow automatic acquisition of multiple images at defined intervals. These images were then stitched together using a second program called "Scion Image". A "montage" function exists in the Scion image to stitch the images together to reconstruct the full picture.

After the arrays are manufactured, the DNA probes must be generated. RNA samples were extracted from cell lines or tissues using standard molecular biology protocols. Typical probe labeling procedures involved the reverse transcription of the RNA sample to obtain cDNA followed by two PCR amplification steps. A size-selection step by gel electrophoresis was used to select PCR products within a 170-200bp range (tk insert + primers). The amplified PCR products were then used in random-primed labeling reaction incorporating Cy3-dUTP and Cy5-dUTP. A 200µl PCR reaction was sufficient to produce DNA for 10 labelings. Hybridizations were performed in an enclosed chamber under a 12mm diameter coverslip and placed in oven overnight at 65ºC. The following day, hybridized arrays were subjected to three washes at increasing stringency. The wash conditions were optimized to obtain a maximum signal to background ratio. An Axon GenePix® 4000A scanner and its software GenePix Pro 3.0 were used for image acquisition and quantitative analysis. Data obtained from GenePix Pro 3.0 in the form of tab-delimited text was imported into Microsoft Excel® or other statistical software for further analysis. Up- or down-regulated genes were distinguished or grouped together by their Cy5/Cy3 ratios after normalization.

3. Tk gene expression in breast cancer specimens and cell lines

RNAs for our studies were derived from cell lines and breast cancer tissues. Initially, cell lines of human mammary origin were used to optimize the hybridization protocols since these cell lines might approximate RNA expression patterns of normal or tumor tissues. Large quantities of RNA can be prepared from these cell lines for repeated experiments. We collaborated with two breast cancer biologists (at LBNL and UCSF, respectively) to test our tk gene arrays using well characterized cell lines. For example, one test involved hybridizing probe cDNA derived from an RNA sample from the breast cancer-derived cell line MCF7 and comparing it to probe cDNA derived from RNA from normal breast tissue. The oncogene erbB2 is known to be amplified in many breast cancer patients and breast tumor-derived tissue cultures. In our experiment, the expression level of the erbB2 transcript is high compared to that in normal breast tissue (Figure 1A). The experiments demonstrated the tk array could confirm previous observations and provide a different insight into changes underlying tumor development.

A second example compared tk gene expression in a mammary gland-derived cell line (184A1) against a derivative that was transformed with large T antigen to form tumors (184A1TH)(Figure 1B). The hybridization results indicated that the transformation induced a significant up- and down-regulation of several tyrosine kinase genes, among them a epherin receptors. This came as no surprise since previous experiments in other laboratories suggested that the over-expression of EphA2 as well as down-regulation of EphA4 might be involved in malignant transformation. Our hybridization results are, again, in agreement with previous observations.

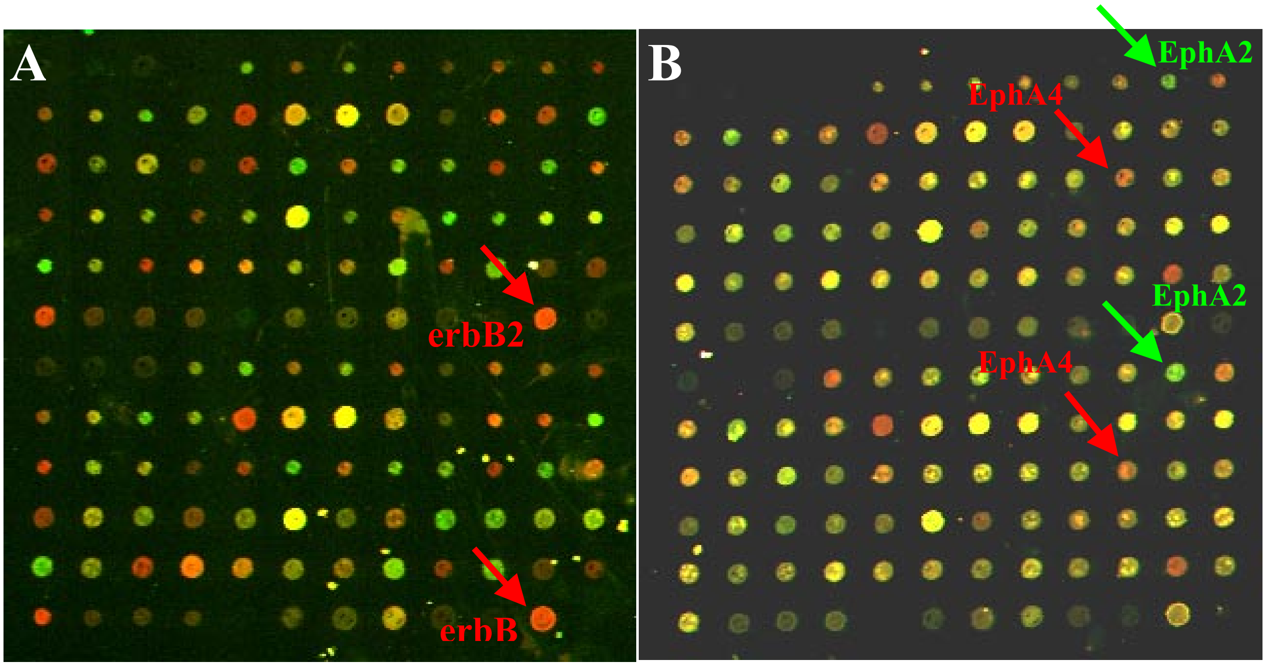

Figure 1.

A. Strong expression of erbB2 gene is seen as a bright red spot when probe derived from RNAs of a breast cancer-derived cell line MCF7 (shown in red) was co-hybridized with probe derived from RNAs of a normal breast tissue sample (shown in green).

B. Expression level change involved in anchorage-independent growth and tumor metastasis: Mammary gland-derived cell line 184A1 (shown in red) was co-hybridized with its transformed (with large T antigen) counterpart 184A1TH (shown in green). The up- (EphA2) and down- (EphA4) regulation of the epherin receptors correlates with a hypothesis on the biological model system.

B. Training received through the fellowship and its significance for the recipient

1. Cell and molecular biology techniques acquired

In the course of experiments, various cell and molecular biology techniques were acquired. These included mammalian cell culture, RNA preparation and quantitation, RT-PCR, DNA labeling as well as optimization of hybridization protocols.

2. Training to further understand the mechanism of cancer

Through studying the association of multiple genetic markers with the development and progression of cancer, we learned a lot about the complexity of the disease. It would be difficult, if not impossible, to conclude any meaningful results without a sound model system. After years of dedicated research, our collaborators have established excellent models for breast cancers, which allow us to apply our DNA microarray system to demonstrate the feasibility of our approach. The experience of collaborating with these excellent cancer biologists has helped Dr. Hsieh tremendously in understanding the mechanisms of breast cancer.

3. Course attended at University of California, Berkeley

As the number of successful hybridizations increased, the amount of data gathered from microarray experiments exploded. To efficiently analyze the data, a good command of statistical skill for data analysis is required. Dr. Hsieh took a statistics class at the University of California at Berkeley in the autumn semester of 2000 to learn the fundamental techniques in data analysis.

4. Workshops attended

As an integral part of fellowship training, Dr. Hsieh completed a special course in DNA microarray, functional genomics, proteomics, and bioinformatics held by the Biotechnology Program at the University of California, at Davis in 1999. The 3-day lecture and 2-day hands-on bioinformatics workshop offered practical, as well as informative, training in DNA microarray knowledge and database theory. To broaden the knowledge in applications of microarray technology, Dr. Hsieh also attended a 5-day workshop sponsored by the Institute of Pure and Applied Mathematics of the University of California at Los Angeles in November of

2000. Thanks to the sponsorship of the UC BioStar program, the registration and travel expenses were partially compensated.

5. Travel awards

During the fellowship training, Dr. Hsieh received two travel awards from the Histochemical Society (HCS) and International Congress of Histochemistry and Cytochemistry (ICHC) to attend their conferences and present his works. The first conference was the Histochemical Society 51st Annual Meeting which took place on March 24-26, 2000 in New Orleans. Dr. Hsieh presented his work in form of a poster entitled "Expression Profiling of Tyrosine Kinase Genes by FISH and Chips". Following the annual meeting, the executive secretary of the HCS awarded him a travel award to attend the 11th ICHC Congress held in York, United Kingdom, on Sept 3-8, 2000.

6. Significance of the fellowship to the recipient

This CRFA fellowship has enabled the recipient to participate in a project focussing on utilizing innovative technology to identify DNA markers for breast carcinogenesis. Previously, Dr. Hsieh was trained in biochemical engineering. The training effort supported by the fellowship provided an opportunity for Dr. Hsieh to contribute his expertise in the field of system integration. Collaboration with a cell biologist and a biophysicist in the group was an invaluable learning experience. After exploring the importance of tyrosine kinases in signal transduction and their regulatory roles in tumor origin and progression, he believed further discoveries in the regulatory patterns of these tk genes could contribute tremendously to the understanding of cancer development and progression. This understanding and the availability of diagnostic tools, such as the DNA microarray, would eventually allow the screening of cancer markers at the earliest stage, and hopefully, contribute to the ultimate goal of early cancer detection and prevention.

C. Public presentations of sponsored work

1. Conference presentations

1. **H.-Ben Hsieh**, Robert A. Lersch, Daniel E. Callahan, Simon Hayward, Mariwil Wong, Orlo H. Clark, Heinz-Ulrich G. Weier. "Monitoring signal transduction in cancer: DNA microarray for semi-quantitative analysis", The Histochemical Society 51st Annual Meeting, Santa Fe, NM, February 2001.
2. **Hsieh, H.B.** and Weier, H.-U. G. "Kinase Gene Expression Profiling by FISH and Chips", The 11th International Congress of Histochemistry and Cytochemistry, York, United Kingdom, September 2000.
3. **Hsieh, H.B**., Weier, H.-U.G., Kinase Gene Expression Profiling in Human Tumors", Department of Subcellular Structure Seminar, Life Sciences Division, E.O. Lawrence Berkeley National Laboratory, May 10, 2000.
4. **Hsieh, H.B**., Weier, H.-U.G., Kinase Gene Expression Profiling-Instrumentation & Prototype Setup", Oral presentation in Corning Inc., Corning, N.Y., May 8, 2000.
5. **Hsieh, H.B**., Weier, H.-U.G. "Expression Profiling of Tyrosine Kinase Genes by FISH and Chips", The Histochemical Society 51st Annual Meeting, New Orleans, LA, March 24-26, 2000.
6. **Hsieh, H.B**. and Weier, H.-U.G. "DNA Microarray for Gene Expression Profiling of Human Tumors", Seminar in Academia Sinica, Taiwan, January. 6, 2000.

2. Journal Publications

Various aspects of the work performed in the period of support have been published in peer-reviewed journals. Copies of these publications are attached.

1. **H.-Ben Hsieh**, Robert A. Lersch, Daniel E. Callahan, Simon Hayward, Mariwil Wong, Orlo H. Clark, Heinz-Ulrich G. Weier. "Monitoring signal transduction in cancer: DNA microarray for semi-quantitative analysis", *Journal of Histochemistry and Cytochemistry* (2001) 49:1057-1058.
2. Heinz-Ulrich G. Weier, Horst F. Zitzelsberger, **H.-Ben Hsieh**, Melita V. Sun, Mariwil Wong, Robert A. Lersch, Paul Yaswen, Jan Smida, Christine Kuschnick and Orlo H. Clark. "Monitoring signal transduction in cancer: tyrosine kinase gene expression profiling", *Journal of Histochemistry and Cytochemistry* (2001) 49: 673-674.
3. Robert A. Lersch, Jingly Fung, **H.-Ben Hsieh**, Jan Smida, Heinz-Ulrich G. Weier, "Monitoring signal transduction in cancer: from chips to FISH", *Journal of Histochemistry and Cytochemistry* (2001) 49: 925-926.
4. H.U. Weier, J. Fung, S. Munne, R.A., Lersch, **H.B. Hsieh**, X.N. Chen, J.Korenberg, R.A Pederson. "Towards a Full Karyotype Screening of Interphase Cells: 'FISH and Chip' Technology", *The American Journal of Human Genetics* (2000), V67 (Suppl.), A166.
5. **H.-P. B. Hsieh**, M. Wang, R.A. Lersch, U.-J. Kim, H.-U. G. Weier, "Rational Design of Landmark Probe for Quantitative DNA Fiber Mapping", *Nucleic Acids Research* (2000) 28 (8): e30.
6. **Huangpin B. Hsieh** and Heinz-Ulrich G. Weier, "Expression Profiling of Tyrosine Kinase Genes by FISH and Chips", The Journal of Histochemistry and Cytochemistry (2000) 48 (12):1729.
7. Weier, H.-U.G., Munné, S., Lersch, R.A., **Hsieh, H.B.**, Smida, J., Chen, X.-N., Korenberg, J.R., Pedersen, R.A., Fung J. (2001) Towards a Full Karyotype Screening of Interphase Cells: 'FISH and Chip' Technology. Molecular and Cellular Endocrinology 183:41-45

3. Publication of Award

The award was published in LBNL's newletter 'Currents' (see attachement).

B. Funding received as a result of the supported training activity

We have been able to significantly leverage funding provided by CRFA. The support provided by the CRFA was instrumental in generating preliminary data which allowed us to compete successfully for subsequent funding. The following grants were obtained as a result of the activities partially supported by the fellowship.

1. U.S. Army Medical Research and Materiel Command, Prostate Cancer Research Program, 'Tyrosine Kinase Gene Expression Profiling in Prostate Cancer', $224,775 direct cost.
2. California Cancer Research Program (New Investigator Award), 'Tyrosine Kinase Expression Profiling in Prostate Cancer', $121,720 direct cost.
3. NCI, 'Spectral Imaging for Phenotype Analysis of Tumor Cells', $329,275 direct cost.
4. NCI, 'Spectral Imaging for Phenotype Analysis of Tumor Cells (Supplement)', $262,140 direct cost.